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Short Report: Dual Infection of Novel Influenza Viruses A/H1N1 and A/H3N2 in a Cluster of Cambodian Patients

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Abstract. During the early months of 2009, a novel influenza A/H1N1 virus (pH1N1) emerged in Mexico and quickly spread across the globe. In October 2009, a 23-year-old male residing in central Cambodia was diagnosed with pH1N1. Subsequently, a cluster of four influenza-like illness cases developed involving three children who resided in his home and the children's school teacher. Base composition analysis of internal genes using reverse transcriptase polymerase chain reaction and electrospray ionization mass spectrometry revealed that specimens from two of the secondary victims were coinfecting with influenza A/H3N2 and pH1N1. Phylogenetic analysis of the hemagglutinin genes from these isolated viruses showed that they were closely related to existing pH1N1 and A/H3N2 viruses circulating in the region. Genetic recombination was not evident within plaque-purified viral isolates on full genome sequencing. This incident confirms dual influenza virus infections and highlights the risk of zoonotic and seasonal influenza viruses to coinfect and possibly, reassort where they cocirculate.

Globally, influenza remains a leading cause of human morbidity and mortality, largely as a result of the virus's inherent evasiveness from the immune response.¹ Coinfection of viruses in birds or mammals, such as swine, increases the chance for the emergence of new variants.^{2,3} Novel viruses can emerge within a population, evade immunity, and result in local epidemics or in some instances, pandemics. However, recombination among subtypes remains rare.⁴ In early 2009, a novel influenza A/H1N1 virus (pH1N1) emerged in Mexico. By October 2009, pH1N1 had become the predominant influenza subtype infecting populations in most areas of the world.⁵ Notwithstanding, in Southeast Asia, seasonal influenza viruses as well as the avian influenza virus A/H5N1 continued to circulate [World Health Organization (WHO) Pandemic (H1N1) 2009–Update 82; http://www.who.int/csr/don/2010_01_08/en/index.html]. In the Southeast Asian nation of Cambodia, we and others have shown that cases of influenza peak with the monsoon between the months of July and December.^{6,7}

In early October 2009, a 23-year-old man from central Cambodia presented to the Ta Khmau health clinic with influenza-like symptoms (Table 1). Real-time reverse transcriptase polymerase chain reaction (rRT-PCR) assays to detect influenza A and B viruses were used to diagnose pH1N1 infection.⁸ The patient received treatment to alleviate symptoms and recovered at his residence.

On October 14, 2009, three male (M) children, ages 13, 8, and 4 years, who lived in the same home as the suspected index case presented at the health clinic with fever (39°C), cough, sore throat, headache, and symptoms of either nausea or vomiting (Table 1). The students attended classes in a single-room school. They reported neither recent extended contact with animals nor travel. A day after their illness, their teacher reported febrile illness, and samples were obtained. Specimens from one of three school children and the teacher indicated dual infection with both seasonal A/H3N2 and pH1N1 viruses.

Virus isolation from collected clinical specimens was performed in Madin-Darby canine kidney (MDCK) cells and

shell vials. Isolated viruses were analyzed on the Ibis T5000 by ESI-MS (Ibis Biosciences, Inc., Carlsbad, CA) analysis to generate a specific mass measurement for each amplified PCR product. Primer sequences and other PCR components were as previously described.⁹ The base composition signature for the product was then compared with known sequences in a database to generate an internally verified identification. Analyses from six regions of the influenza genome confirmed pH1N1 infection in the 23-year-old man and dual A/H3N2 and pH1N1 infection in specimens from one of three children (8/M) and the teacher (24/female [F]). Only A/H3N2 viruses were evident in samples from the 4/M and 13/M victims. Analysis did not discern recombined signatures in gene segments within the isolated viruses (Table 2).

To fully characterize the gene segments of dual-infected individuals, single-passaged viruses from two of the patients and six of corresponding purified virus plaques were processed for pyrosequencing using a specialized multisegment RT-PCR procedure to amplify the genome of all subtypes of the influenza A virus through degenerate primers. Sequencing, genome assembly, and closure reactions were performed as previously described.¹⁰ Complete genomes (> 99% open reading frame [ORF]) were obtained for all eight segments of each virus isolate. A complete ORF region (100% genome length) was obtained for all isolates. Sequences for the hemagglutinin (HA) segment from the isolates were compared with known sequences (data not shown). Relative to A/Perth/16/2009 (H3N2), the H3N2 vaccine component for 2010 and 2011, a total of 3 aa substitutions were seen in the area sequenced, I25V, P162T, and S214I, with an overall similarity of 99%. The latter mutation corresponds to a previously identified antibody combining site.¹¹ More complete datasets for recent swine strains allowed for a fuller comparison of pH1N1 HA sequences. Comparison between the pH1N1 reference strain, A/California/04/2009, and the full genome sequence of isolate material from 4/M revealed 4 aa changes from the vaccine strain (P100S, S220T, I338V, and Y528H), with an overall similarity of 99.3% over 566 aa. Phylogenetic analysis of the HA genes of all analyzed plaques revealed a single genetic sequence for both the A/H3N2 and pH1N1 strains. None of the isolated plaques showed evidence of recombination between

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TABLE 1
Demographics of Cambodian cases involved in influenza cluster

	FSS08728	FSS08731	FSS08732	FSS08733	FSS09305
Age (years)/sex	23/M	13/M	8/M	4/M	24/F
Disease onset	10/08/09	10/12/09	10/12/09	10/13/09	10/12/09
Date sampled	10/09/09	10/14/09	10/14/09	10/14/09	10/15/09
Occupation	Lawyer	Student	Student	Student	Teacher
Clinical findings*					
Fever (°C)	39°C	39°C	39°C	39°C	39°C
Malaise	Y	N	N	N	Y
Headache	Y	Y	Y	Y	Y
Sore throat	Y	Y	Y	Y	N
Cough	Y	Y	Y	Y	Y
Nausea	N	Y	N	N	N
Vomiting	N	N	Y	Y	N
Rhinorrhea	N	N	N	N	Y
Medication	Amoxicillin and paracetamol	Amoxicillin and paracetamol	Amoxicillin and paracetamol	Amoxicillin and paracetamol	N
Epidemiology					
Recent travel	Within country	N	N	N	N
Animal exposure	N	N	N	N	N
Disposition	Recovered	Recovered	Recovered	Recovered	Recovered

* Recorded on date of sample collection.

pH1N1 and A/H3N2, and all had full sequences for the eight influenza segments from both strains.

Herein, we describe a cluster of influenza-like illness (ILI) cases at a school in central Cambodia. Among the afflicted, two were coinfecting with A/H3N2 and pH1N1 influenza viruses. The finding of coinfections has rarely been reported. A recently study of over 2,000 clinical samples found no dual infection.¹² However, coinfection of pH1N1 and A/H3N2 has been reported in a 38-year-old woman from Singapore,¹³ and mixed infection was also evident in six individuals after an outbreak of influenza at a college campus near Beijing, China.¹⁴ A more recent New Zealand study collected and screened 1,044 clinical samples during the pandemic and found 11 coinfections with A/H1N1 seasonal viruses.¹⁵

Transmission of pH1N1 at a time when seasonal influenza viruses were circulating in Cambodia resulted in coinfection and raised the possibility of reassortment. The generation of novel influenza viruses through reassortment has occurred when zoonotic viruses mix in birds, swine, and humans, and gene segments are reshuffled. Pandemic strains often are the result of emerging viruses from reservoirs to which humans have little or no immunity. The A/H2N2 1957 and A/H3N2 1968 pandemics occurred after reassortment between human and avian strains.^{16,17} The 1957 virus was generated when A/H1N1 1918 reassorted with avian viruses to pick up new PB1, HA, and neuraminidase (NA) segments. Similarly, the novel virus

isolated from ILI cases in southern California in April 2009 contained genetic elements from four different sources, including North American swine influenza viruses, North American avian influenza viruses, human influenza viruses, and a Eurasian swine influenza virus.¹⁸ In our analysis, recombination was not detected in viruses isolated from the Cambodian patients.

The clinical disease within the dual A/H3N2 and A/H1N1 Cambodian patients did not result in hospitalization nor did these patients' disease seem more severe than the disease in the other patients with influenza.¹⁹ Clinical findings were broad, including upper respiratory and gastrointestinal symptoms. None of five patients in this outbreak had been vaccinated against either seasonal or pH1N1 influenza infections. Indeed, in rural Cambodia, little seasonal influenza vaccination is conducted, and use of therapeutics such as neuraminidase inhibitors is rare²⁰; thus community-wide immunity is lacking.

Southeast Asia has proven to be a critical region for the adaptation and emergence of variants of seasonal influenza viruses²¹ as well as an area of zoonotic virus transmission in humans. Cases of A/H5N1 have largely been restricted to the Near East and southeast Asia, with Cambodia suffering 15 confirmed human cases and 13 fatalities since 2005. The endemicity of A/H5N1 in poultry in many areas of south-east Asia provides increased opportunity for human exposure and adaptation of a lethal virus suitable for sustained human transmission. Our findings emphasize the importance

TABLE 2
Base composition data from clinical samples

Patient	Detection*	Target segment					
		PB1	NP	M1	PA	NS1	NS2
23/M	1:pH1n1	A39 G32C24 T33	A35 G21 C20 T25	A24 G28 C24 T29	A36 G25 C27 T24	A36 G36 C20 T28	ND†
13/M	1:H3N2	A41 G30C23 T34	A32 G24 C20 T25	A25 G29 C21 T30	A31 G25 C25 T31	A40 G30 C20 T29	A37 G25 C16 T27
8/M	1:pH1N1	A41 G30 C22 T35	A35 G21 C20 T25	A25 G27 C24 T29	A37 G24 C26 T25	A37 G34 C19 T29	A35 G28 C15 T27
8/M	2:H3N2	A41 G20 C23 T34	ND	A25 G29 C21 T30	ND	A41 G29 C19 T30	A37 G25 C16 T27
4/M	1:H3N2	A41 G30 C23 T34	A32 G24 C20 T25	A25 G29 C21 T30	A41 G22 C26 T23	A40 G30 C20 T29	A37 G25 C16 T27
24/F	1:pH1Ni	ND	A35 G21 C20 T25	A25 G27 C23 T30	ND	A37 G34 C19 T29	A34 G29 C16 T26
24/F	2:pH3N2	A40 G31 C23 T34	ND	A25 G29 C21 T30	A40 G23 C27 T22	ND	A37 G25 C16 T27

ND = not determined.

* The listing of strain determinations made by the PlexID instrument based on the base compositions detected in the sample.

† The more recent version of the influenza surveillance plate that was used for sample 23/M does not include an NS2 primer pair.

of national and international cooperation to survey for the emergence of novel and/or reassorted influenza viruses.

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